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Cell Density and Cell Aging as Factors Modulating Antifungal Resistance of *Candida albicans* Biofilms[▽]

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Biofilm formation is a major virulence attribute of *Candida* pathogenicity which contributes to higher antifungal resistance. We investigated the roles of cell density and cellular aging on the relative antifungal susceptibility of planktonic, biofilm, and biofilm-derived planktonic modes of *Candida*. A reference and a wild-type strain of *Candida albicans* were used to evaluate the MICs of caspofungin (CAS), amphotericin B (AMB), nystatin (NYT), ketoconazole (KTC), and flucytosine (5FC). Standard, NCCLS, and European Committee on Antibiotic Susceptibility Testing methods were used for planktonic MIC determination. *Candida* biofilms were then developed on polystyrene wells, and MICs were determined with a standard 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide assay. Subsequently, antifungal susceptibility testing was performed for greater inoculum concentrations and 24- and 48-h-old cultures of planktonic *Candida*. Furthermore, *Candida* biofilm-derived planktonic cells (BDPC) were also subjected to antifungal susceptibility testing. The MICs for both *C. albicans* strains in the planktonic mode were low, although on increasing the inoculum concentration (up to 1×10^8 cells/ml), a variable MIC was noted. On the contrary, for *Candida* biofilms, the MICs of antifungals were 15- to >1,000-fold higher. Interestingly, the MICs for BDPC were lower and were similar to those for planktonic-mode cells, particularly those of CAS and AMB. Our data indicate that higher antifungal resistance of *Candida* biofilms is an intrinsic feature possibly related to the biofilm architecture rather than cellular density or cellular aging.

Candida is by far the most important fungal pathogen of humans, causing diseases varying from superficial mucosal infections to life-threatening systemic disorders (34). *Candida* infections have been documented as a leading cause of nosocomial infection, with reported mortality rates as high as 40% (6, 41, 42). Further, prevailing in many guises, *Candida* causes significant mortality and morbidity in compromised patient populations such as human immunodeficiency virus/AIDS patients and organ transplant recipients on immunosuppressive therapy (33).

Candida infections primarily begin with adherence and colonization of an artificial or a biotic host surface, leading to the formation of surface-attached communities known as biofilms. These structured communities, encased in a matrix of exopolymeric substances, display unique characteristics that confer survival advantages over their planktonic counterparts (12, 14). Indeed, it has been documented that at least 65% of all microbial infections are related to biofilms (29). *Candida* can develop biofilms on almost all medical devices in current use, including stents, shunts, prostheses (voice box, heart valves, dentures, etc.), implants (lens, breast, etc.), and various types of catheters (31).

The major clinical relevance of *Candida* biofilms, and in fact of all microbial biofilms, is their high resistance to antimicrobials. *Candida* biofilms are known to exhibit elevated antifungal resistance compared to their planktonic counterparts in liquid culture for a number of antifungal agents including amphotericin B (AMB), fluconazole, itraconazole, and ketoconazole (KTC) (3, 11, 16). Some studies, however, suggest that newer antifungal formulations such as echinocandins and liposomal formulations of AMB are much more active against *Candida* biofilms (2, 21).

Although biofilm-forming ability is a major pathogenic attribute of this ubiquitous fungus, its properties including the mechanisms of resistance to antifungals have yet to be defined. Previous workers have postulated factors such as lower metabolic activity, contact-induced gene expression, the presence of an extracellular matrix, and persister cells as possible reasons for biofilm-associated antifungal resistance (for a recent review, see reference 35). In the latter studies, the NCCLS microdilution assay has been commonly used for MIC determination for planktonic-mode *Candida* while alternative colorimetric assays such as the 3-(4-5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) reduction assays have been used by a few for the same purpose (11, 16). On the contrary, colorimetric assays have been the cornerstone of MIC determination for the biofilm mode of *Candida*. According to the current methodology, the planktonic MIC is compared with that for 24- or 48-h biofilms (10, 16, 23, 32).

It is noteworthy, however, that the NCCLS M-27A microdilution assay used conventionally for MIC determination for *Candida* was originally designed for a standard concentration of the planktonic mode of *Candida* (0.5×10^3 cells/ml) (27). Nevertheless, the *Candida* biofilm cell density after 24 or 48 h is far greater than 0.5×10^3 cells/ml and it is tempting to speculate that the reported antifungal resistance of biofilms may be due to the increased cell mass of biofilms. Indeed, in a recent article, a claim has been made

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that cell density does affect the antifungal resistance of *Candida* (28).

Furthermore, there is also an anomaly with regard to the age of the cells subjected to antifungal susceptibility testing; i.e., NCCLS MIC determination for planktonic-mode cells is performed with fresh cultures whereas a 24- or 48-h-old cell population is used for the biofilm mode. Hence, it could be surmised that cellular aging could contribute to acquired higher drug resistance in *Candida* biofilms.

Therefore, the aim of this study was to investigate the roles of cell density and cellular aging on the comparative antifungal susceptibilities of planktonic, biofilm, and biofilm-derived planktonic cells (BDPC) of *Candida*. Furthermore, we also investigated the effects of growth medium and incubation time with drugs on the relative MICs for the planktonic versus the biofilm mode of *Candida*.

MATERIALS AND METHODS

Organisms and growth conditions. Two strains of *Candida albicans*, namely, a reference strain, ATCC 90028, obtained from the archival collection of the Oral Biosciences laboratory of the Faculty of Dentistry, The University of Hong Kong, and a wild-type (WT) clinical strain, BF-1, used previously for biofilm studies in our laboratory, were used for this study (18, 19). The identities of the yeast isolates were confirmed with the commercially available API 32C identification system (BioMérieux, Marcy l'Etoile, France). Both *Candida* strains were subcultured on Sabouraud dextrose agar (SDA; Gibco Ltd., Paisley, United Kingdom) and maintained at 4°C during the experimental period. Culture purity was confirmed periodically by Gram stain visualization and the germ tube test.

Antifungal agents. Five antifungals commonly used to treat oropharyngeal and systemic candidiasis were selected for this study, viz., caspofungin (CAS) from Merck and nystatin (NYT), AMB, KTC, and flucytosine (5FC) from Sigma. Antifungal agents were prepared as previously described (1).

Determination of MICs for planktonic *Candida* cells. First we determined the MICs of the antifungals CAS, NYT, AMB, KTC, and 5FC for two *Candida* strains with the standard NCCLS (now known as the Clinical and Laboratory Standards Institute [CLSI]) criteria (27). Briefly, inocula from 24-h yeast cultures on SDA were standardized to a turbidity equivalent to a 0.5 McFarland standard at 520 nm with a spectrophotometer. The suspensions were further diluted in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, New York, NY) to yield an inoculum concentration of approximately 0.5×10^3 to 2.5×10^3 cells/ml. The MIC assay was performed with 96-well plates (Iwaki, Tokyo, Japan), and each of the *Candida* species was exposed to a double dilution of the aforementioned antifungals. The plates were incubated at 35°C for 48 h to evaluate MICs.

Furthermore, MICs for planktonic *Candida* cells were also determined according to the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) method (13). The EUCAST method uses RPMI 1640 medium supplemented with 2% glucose as the test medium and an inoculum concentration of 0.5×10^5 to 2.5×10^5 cells/ml. Each of these experiments was performed on three different occasions.

Preparation of standard yeast cell suspensions for biofilm studies. *Candida* cells were grown in SDA medium at 37°C for 18 h. A loopful of the yeast was then inoculated into yeast nitrogen base (YNB; Difco) medium supplemented with 50 mM glucose in a rotary shaker at 75 rpm. After overnight broth culture, the yeast cells were harvested in the late exponential growth phase and washed twice with 20 ml of phosphate-buffered saline (PBS; pH 7.2, 0.1 M) prior to use in the biofilm studies.

Biofilm formation. *Candida* biofilms were developed according to a previously published protocol (18, 19). In brief, washed yeast cells were resuspended in YNB medium supplemented with 100 mM glucose and adjusted to an optical density at 520 nm of 0.38 (1×10^7 cells/ml). The standard cell suspension was used immediately to develop biofilms on commercially available presterilized 96-well polystyrene plates (Iwaki, Tokyo, Japan). First, 100 μl of a standardized cell suspension (1×10^7 cells/ml) was pipetted into each well of a microtiter plate and incubated for 1.5 h at 37°C in a shaker at 75 rpm to permit yeast adherence to the well surface (adherence phase). For controls, a well of each microtiter plate was handled in an identical fashion except that no *Candida* suspension was added. Following the adhesion phase, the cell suspensions were aspirated and each well was washed with 100 μl of PBS to remove loosely adherent cells. A 200-μl volume of YNB medium with 100 mM glucose was then pipetted into each of the washed wells, and the plates were incubated at 37°C in a shaker at 75 rpm for 48 h. After the 48-h growth phase, microscopic examination of the cultures was performed to rule out contamination.

Antifungal susceptibility test (AST) for *Candida* biofilms. ASTs of *Candida* biofilms were carried out as previously described (32). After 48 h, the suspending medium was aspirated and biofilms were washed with 100 μl PBS to remove the nonadherent cells. The stock solutions were diluted twofold with RPMI 1640 medium supplemented with 2% glucose to obtain drug concentrations of 100 to 0.1 μg/ml for CAS, 240 to 0.225 μg/ml for AMB, 64 to 0.125 μg/ml for NYT and KTC, and 420 to 0.4 μg/ml for 5FC. A total of 100 μl of the drug solution was added to the microtiter plate containing *Candida* biofilms. Biofilms were then incubated at 37°C for 24 or 48 h with the antifungals, and afterwards the metabolic activity of fungal cells was determined by the XTT reduction assay with minor modifications (18, 32).

XTT reduction assay. The XTT reduction assay used was a modification of the methods described previously (18, 19, 32). Briefly, an XTT (Sigma, St. Louis, MO) solution (1 mg/ml in PBS) was prepared, filter sterilized through a 0.22-μm-pore-size filter, and stored at −70°C. A menadione (Sigma) solution (0.4 mM) was prepared and filter sterilized immediately before each assay. Prior to each assay, the XTT solution was thawed and mixed with the menadione solution at a ratio of 5 to 1 by volume.

The biofilms were first washed three times with 200 μl of PBS, and then 200 μl of PBS-XTT-menadione solution was added to each of the prewashed wells and the control wells. The microtiter plate was then incubated in the dark for 3 h at 37°C. Following incubation, 100 μl of solution was transferred to new wells and the color change in the solution was measured with a microtiter plate reader (SpectraMAX 340 Tunable Microplate Reader; Molecular Devices Ltd., Sunnyvale, CA) at 490 nm. The absorbance values for all new wells were read at 490 nm. The absorbance values for the controls were then subtracted from the values for the test wells to eliminate spurious results due to background interference.

The MIC_{50} for biofilm *Candida* cells was defined as the lowest drug concentration with a 50% reduction in opacity compared with the drug-free control. To exclude the effect of the growth medium on the MIC endpoint for the biofilms, we performed the antifungal assays with two different media, namely, RPMI 1640 medium supplemented with 2% glucose and YNB medium supplemented with 100 mM glucose. Each experiment was performed three times with four replicates on separate occasions.

Determination of cell density in *Candida* biofilm and age-matched planktonic cultures. Since the MICs of all of the antifungals tested were higher for the biofilm mode of *Candida* compared to the planktonic mode, we queried whether this increased resistance was an artifact due to the higher cell density of the biofilms. Therefore, the cell densities of the developing biofilms were assessed by CFU counting and spectrometric analysis as described previously (39). *Candida* biofilms were developed as described above, and cell densities were determined for the 24- and 48-h time points. Cell densities were also determined in parallel for age-matched planktonic cultures that were propagated under identical conditions, and identical initial inocula (10^7 cells/ml) were used for the biofilm experiments.

Drug susceptibility testing with higher-density planktonic suspensions. Since the aforementioned experiment revealed that the cell densities of the 24- and 48-h biofilms were higher than the standard inoculum concentration used for the determination of MICs for planktonic *Candida* by either the NCCLS or the EUCAST method, we surmised that the reported increase in antifungal resistance of biofilm mode cells was due to the increased cell density. To further investigate this possibility, we increased the cell density of the planktonic cultures from 1×10^5 to 1×10^8/ml and conducted ASTs with the broth microdilution assay as described earlier. Classical growth curves could be observed with a cell concentration of 1×10^7/ml for MIC determination. However, once the cell concentration increased beyond 1×10^8/ml, *Candida* suspension growth curves were not clearly discernible due to the very high optical density of the suspensions. Therefore, we repeated the ASTs of higher-inoculum *Candida* suspensions (1×10^7 and 1×10^8 cells/ml) with XTT as described above. Drugs were serially double diluted in 96-well microtiter plates, and high cell density planktonic suspensions were added as described for the NCCLS method. All of the assays were performed in parallel with both RPMI 1640 medium supplemented with 2% glucose and YNB medium supplemented with 100 mM glucose.

Antifungal susceptibility of *C. albicans* SC5314. The first set of experiments described above indicated that, in the biofilm mode, both the reference and WT *C. albicans* strains were resistant to CAS and AMB although the high-density planktonic cultures were susceptible to both drugs. Since a claim has been made in a recent report (28) that, in planktonic mode, *C. albicans* SC5314 drug

TABLE 1. MICs of antifungal agents for the planktonic versus biofilm modes of *Candida*

Drug and strain	MIC (μg/ml)					
	NCCLS, 10^3 cells/ml	EUCAST, 10^5 cells/ml	Planktonic cells, 10^7/ml[a]	Planktonic cells, 10^8/ml[a]	Biofilm, 24 h, 10^7 cells/ml[a]	Biofilm, 48 h, 10^8 cells/ml[a]
CAS						
ATCC	0.2	0.2	0.2	0.2	100	100
WT	0.2	0.2	0.2	0.4	>100	>100
AMB						
ATCC	0.23	0.23	0.23	0.46	15	15
WT	0.23	0.23	0.46	0.93	30	30
NYT						
ATCC	1	1	2	8	16	16
WT	2	2	2	16	32	32
KTC						
ATCC	0.125	0.125	8	64	64	64
WT	0.125	0.125	16	>64	>64	>64
5FC						
ATCC	0.8	0.8	6.5	>420	>420	>420
WT	0.8	0.8	6.5	>420	>420	>420

[a] Determined by XTT reduction assay.

resistance depends on cell density, we repeated the above-described experiments with the same strain, *C. albicans* SC5314, used by the previous workers. Hence, for the AST of planktonic-mode *C. albicans* SC5314, the NCCLS method and high-density cultures (1×10^8 cells/ml) were used. *C. albicans* SC5314 biofilms were formed as described above for 24 h, and cell density was determined by CFU counting and spectrometric analysis. MICs for *C. albicans* SC5314 biofilms were determined with the XTT reduction assay as described above.

Antifungal susceptibility testing of age-matched planktonic cultures. Next, we asked what other factors, apart from cell density, could contribute to the higher antifungal resistance seen in *Candida* biofilms compared to planktonic cultures. Since biofilms are preincubated for 24 or 48 h before the addition of drugs, cell aging may contribute to their antifungal resistance. Furthermore, it has been reported that antifungal-resistant cell populations develop with the maturation of *Candida* biofilms (10). Therefore, to explore this possibility, 1×10^7-cell/ml standard suspensions were prepared as described above for biofilm experiments. These suspensions were divided into two aliquots and propagated in the planktonic and biofilm modes under identical conditions. Along with the biofilm, age-matched planktonic cultures, for 24- and 48-h periods, were evaluated by AST with the aforementioned XTT reduction assay.

Antifungal susceptibility of BDPC. Next, we evaluated whether the high antifungal resistance of *Candida* cells in the biofilm mode of growth is due to a phenotypic or a genotypic alteration of cells. We postulated that if the drug resistance is a genetically acquired trait, then once biofilm cells are resuspended in the planktonic mode, they should maintain the antifungal resistance acquired in the biofilm mode. To evaluate this hypothesis, we grew *Candida* biofilms as mentioned above. After 24 and 48 h, the biofilms were washed gently and scraped and pipetted into a new 96-well microtiter plate containing serially double-diluted drugs as described earlier. These BDPC were incubated with all of the antifungals tested for either 24 or 48 h, and MICs were determined by XTT reduction assay as described earlier.

RESULTS AND DISCUSSION

Biofilm formation is a major virulence attribute of *Candida*. Since the pioneering work of Hawser and Douglas in 1994 (16), higher antifungal resistance of *Candida* biofilms has been shown by a number of other investigators (10, 23, 32). However, in these studies, the MICs of antifungals for the biofilm mode of *Candida* derived from colorimetric assays such as the XTT reduction assay were compared with those of planktonic-mode *Candida* cells by using MICs derived by the NCCLS microdilution method. The latter method uses planktonic-mode *Candida* suspensions at concentrations of 1×10^3 to 1×10^4 cells/ml for the antifungal assay. In contrast, the biofilm mode *Candida* population used in the XTT reduction assay has a much higher density. This casts doubt on the possibility that the higher cell density in the biofilm mode is a contributory factor in the higher MICs for the *Candida* biofilm mode, as recently claimed by Perumal et al. (28).

Antifungal susceptibility of planktonic and biofilm mode *Candida* by standard methodology. In the first set of experiments in the present study, standard ASTs, i.e., the NCCLS and EUCAST methods, were used to evaluate the MICs for the *C. albicans* ATCC and WT strains. The MICs of all five antifungals (viz., CAS, NYT, AMB, KTC, and 5FC) were lower for both the ATCC and WT planktonic-phase *C. albicans* strains compared with biofilm phase cells when evaluated by either the NCCLS or the EUCAST method (Table 1). Thus, our results are in agreement with previous reports that biofilms are more resistant to antifungals. However, CAS, which has been reported to be effective against the *Candida* biofilm mode (2, 21, 38), was not particularly active against *Candida* biofilms in our study, confirming the observations that the susceptibility of *Candida* biofilms to CAS is variable, possibly due to resistant strains (17, 37).

Antifungal susceptibility of high-density *Candida* planktonic suspensions. Once *Candida* cell densities were determined after the adhesion phase (i.e., 1.5 h), it was found that approximately 10^5 cells/ml remained in each well of a 96-well plate from an initial standard inoculum of 1×10^7 cells/ml. Subsequently, biofilm cell densities reached approximately 1×10^7 and 1×10^8/ml after 24 and 48 h, respectively (Fig. 1). When we repeated the AST of planktonic-mode cells with a greater inoculum concentration of 1×10^8 cells/ml, up to 8-, 256-, and >1,000-fold increased MICs of NYT, KTC, and 5FC, respectively, were noted (Table 1). However, the MICs of CAS and

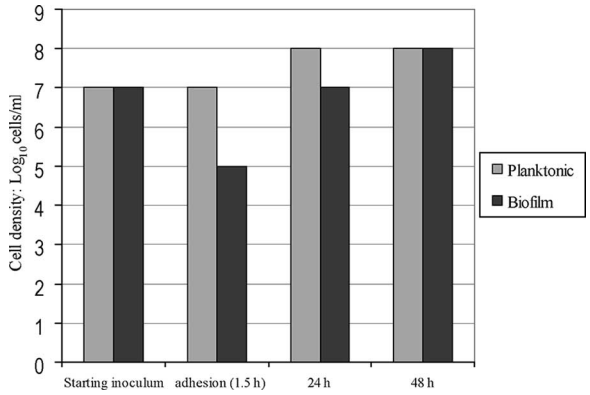

FIG. 1. Histogram illustrating the relative variations in cell density of *Candida* biofilms during maturation and of age-matched planktonic cultures. Note the reduction in the initial inoculum concentration of 1×10^7 to 1×10^5 cells/ml in the adhesion phase due to the removal of nonadherent cells.

AMB did not change significantly, even with the higher inoculum concentration of planktonic *Candida*. Thus, the MICs of the latter two drugs were either similar to or one- to twofold greater than the MICs at the lower inoculum concentration. For instance, the MICs of CAS for the *C. albicans* WT strain at 1×10^8 and 1×10^5 cells/ml were 0.4 and 0.2 μg/ml, respectively (Table 1). Hence, it appeared that the resistance of planktonic-mode *Candida* may depend on the cell density of the suspensions under some circumstances, in particular, for fungistatic drugs such as KTC and 5FC.

However, as shown above, although the higher inoculum concentrations of planktonic *Candida* were susceptible to fungicidal drugs such as CAS and AMB, their biofilm counterparts having an equivalent cell density were resistant to the latter drugs. Therefore, we could conclude that the higher antifungal resistance seen in the *Candida* biofilm mode compared to the planktonic mode is not solely due to the higher cell density. It therefore appears that the nature of the antifungal plays a critical role in determining the relative resistance of the biofilm versus planktonic-mode *Candida*.

Our data confirm results of previous studies which showed that high-density (10^7 cells/ml) planktonic-mode *Candida* cells were susceptible to antifungals whereas biofilm mode cells were resistant (4, 11). In contrast, Perumal et al. (28) recently claimed that cell density is a major factor contributing to the higher antifungal resistance of *Candida* biofilms. The latter workers observed that high-density planktonic cultures of *C. albicans* SC5314 (equal to biofilm cell concentrations) showed a resistance to CAS and AMB similar to that of biofilm mode cells.

Therefore, to explore the possibility of a strain variation effect on antifungal susceptibility, we used the same *C. albicans* strain, SC5314, for the next set of ASTs (24). When we compared the antifungal MICs obtained by the NCCLS method for the planktonic mode with those obtained for the biofilm mode, higher MICs of all of the antifungal tested, i.e., CAS, AMB, KTC, and 5FC, were observed (Table 2). However, once the planktonic-mode *C. albicans* SC5314 concentration was increased to 1×10^8 cells/ml, it was observed that the cells were resistant to KTC and 5FC but not to CAS and AMB (Table 2; Fig. 2 and 3). Hence, our data on CAS and AMB are in line with previous studies (4, 11) but contradict the observations reported by Perumal et al. (28). Nevertheless, our data on KTC are in agreement with the observation of Perumal et al. (28) that high-density planktonic-mode *C. albicans* cells are tolerant of high azole drug concentrations. These discrepancies in results could be due to the modified experimental protocols used in the studies. Therefore, it is imperative that standard protocols be used by future workers in order to understand the enigma of the antifungal resistance of *Candida* biofilms. Considering the data from the present and other, related, studies, we could conclude that although the higher cell density seen in the biofilm mode may partially contribute to antifungal resistance, there are other, as-yet-undiscovered, factors which modulate this phenomenon. These factors may include the nature of the antifungal, as well as contact-induced gene expression or differentially expressed protein biomarkers related to the surface-attached biofilm mode (26, 35, 36).

Another interesting observation of the present study is the paradoxical effect shown by *C. albicans* strains for CAS (Fig. 2). Thus, growth of *C. albicans* could be observed at drug concentrations higher than the MIC, i.e., 12.5 to 50 μg/ml. This

TABLE 2. MICs determined by the NCCLS method and AST of planktonic-mode and 24-h biofilm *C. albicans* SC5314 cells

Drug	MIC (μg/ml)		
	NCCLS[a]	Planktonic mode[b]	24-h biofilm[c]
CAS	0.2	0.2	>100
AMB	0.23	0.46	32
KTC	0.125	>64	>64
5FC	0.8	>420	>420

[a] 10^3 cells/ml.
[b] 10^8 cells/ml.
[c] 10^7 cells/ml.

TABLE 3. Comparative MICs of antifungal agents for 24- and 48-h planktonic, biofilm, and biofilm-derived planktonic *Candida* cells

Drug and strain	MIC (μg/ml)					
	Planktonic cells		Biofilm cells		BDPC	
	24 h	48 h	24 h	48 h	24 h	48 h
CAS						
ATCC	0.2	0.4	100	100	0.2	0.4
WT	0.4	0.4	>100	>100	0.4	0.2
AMB						
ATCC	0.46	0.46	16	16	0.46	1.87
WT	0.93	0.93	32	32	1.87	3.75
NYT						
ATCC	2	8	16	16	2	8
WT	4	16	32	32	4	16
KTC						
ATCC	8	>64	>64	>64	>64	>64
WT	16	>64	>64	>64	>64	>64
5FC						
ATCC	>420	>420	>420	>420	>420	>420
WT	>420	>420	>420	>420	>420	>420

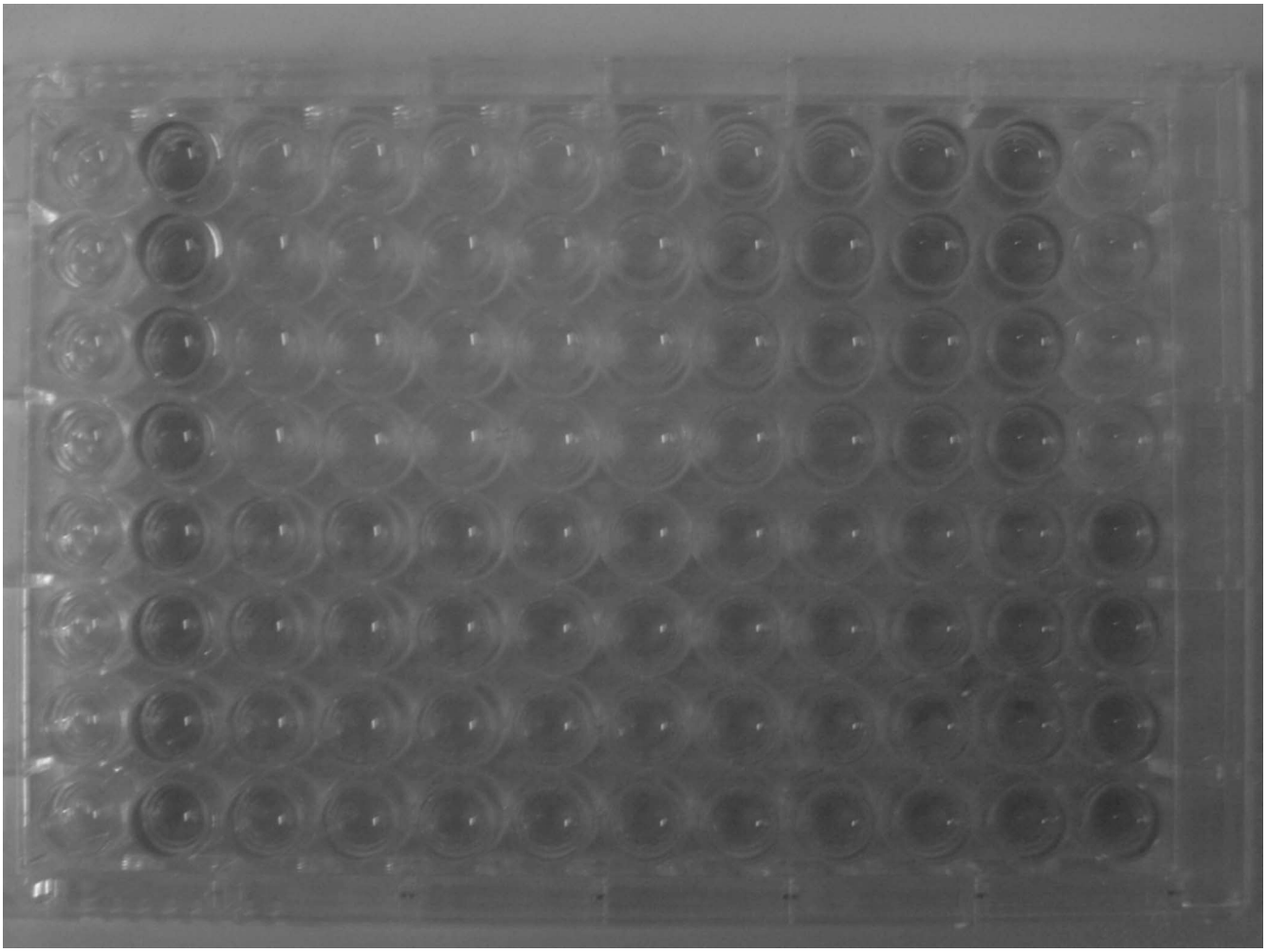

FIG. 2. XTT reduction assay of resistance of high-density (10^8 cells/ml) planktonic cultures of *C. albicans* SC5314 and WT BF-1 to CAS (lines 1 to 4) and AMB (lines 5 to 8). Note the paradoxical effect of *C. albicans* strain resistance to CAS at higher concentrations (12.5 to 50 μg/ml).

observation is in agreement with previous reports on the paradoxical growth of *C. albicans* in the presence of echinocandin drugs (9, 25). Although several hypotheses have been proposed to explain this phenomenon, the exact mechanism(s) has yet to be found.

Antifungal susceptibility of age-matched planktonic cells and biofilms. To evaluate the effect of cell aging on MICs, we performed relative antifungal susceptibility testing with age-matched biofilm and planktonic-mode *Candida* cells kept under identical conditions for up to 24 and 48 h (age matched refers to the incubation period for which two growth modes of *Candida* were propagated; however, it should be noted that, due to the disparate growth rates of the biofilm and planktonic-mode cells, the quality of the resultant cell populations at 24 or 48 h is unlikely to be the same). When an AST of the aforementioned age-matched planktonic cultures at 24 or 48 h was performed, we found a MIC profile very similar to that of high-density planktonic cultures (Table 3). For instance, higher MICs of the fungistatic drugs KTC and 5FC and lower MICs of CAS and AMB were noted. As the cell density of planktonic cultures increases after 24 or 48 h, this may have been a contributory factor in the lower susceptibility to fungistatic drugs. In contrast, the MICs of CAS and AMB for respective age-matched biofilm mode cells of *Candida* were relatively higher (Table 3). It is known that aging *Candida* cells or cultures enter into a stationary phase and, in doing so, acquire a degree of resistance to antifungals (7, 8, 40). However, our data indicate that the antifungal susceptibility of aged cultures varies, depending on the antifungals as well as the growth mode (Table 3). Taken together, it could be surmised that factors other than cellular aging play a role in the higher antifungal resistance of *Candida* cells in the biofilm mode.

Effect of culture medium and incubation period on antifungal susceptibility. In order to determine whether the difference in the medium or incubation time used could affect the relative MICs of drugs for planktonic versus biofilm mode *Candida* cells, we used two different media and incubation times for our AST. We noted that for planktonic and biofilm cells, incubation for either 24 or 48 h with the antifungals tested yielded almost identical results (data not shown). Furthermore, incubation in RPMI 1640 medium with 2% glucose or YNB medium with 100 mM glucose had no effect on the MICs for

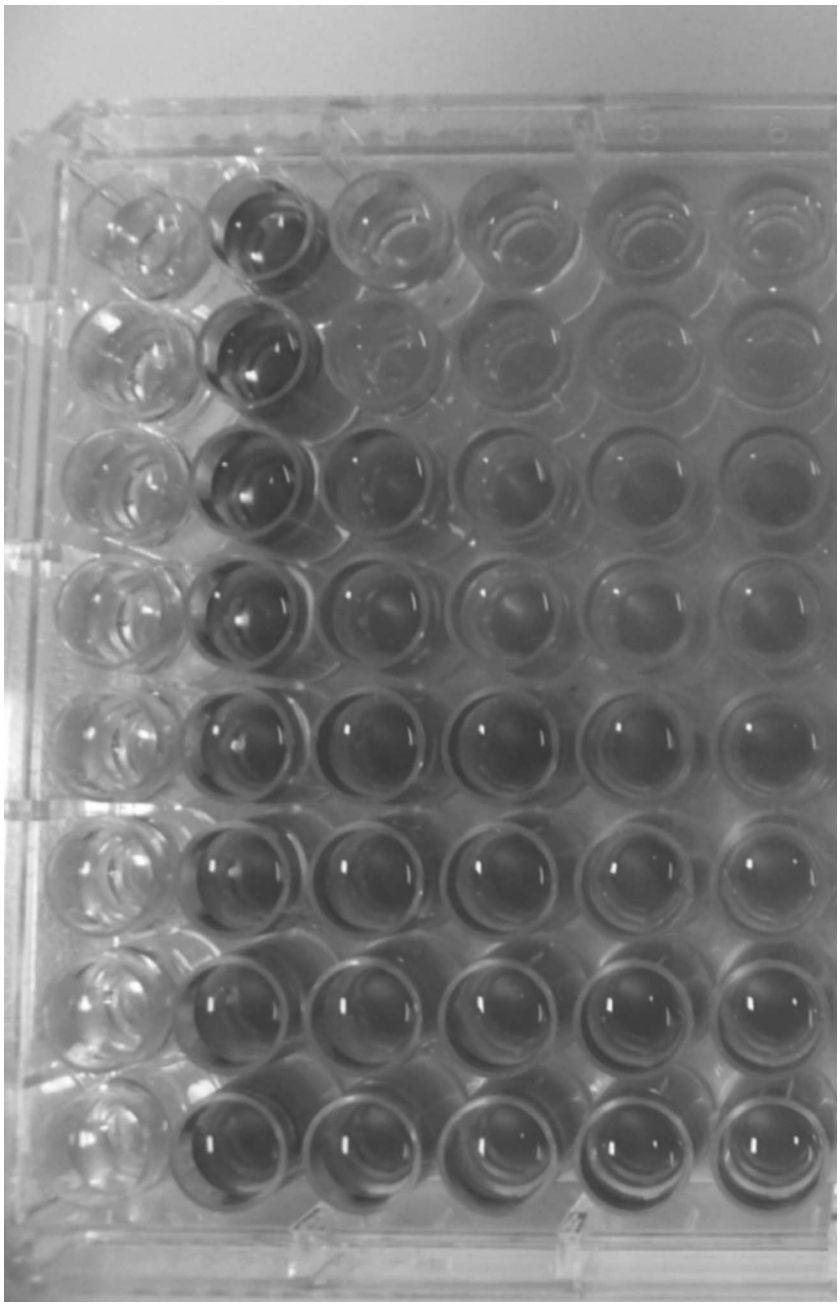

FIG. 3. XTT reduction assay of resistance of high-density (10^8 cells/ml) planktonic cultures of *C. albicans* SC5314 to CAS (lines 1 and 2), AMB (lines 3 and 4), KTC (lines 4 to 6), and 5FC (lines 7 and 8).

either planktonic or biofilm cells (data not shown). It should be noted that, on occasion, the culture medium and incubation period resulted in a deviation of the MIC by 1 dilution. Hence, we could exclude the quality of the culture medium used or the incubation period as a possible contributory factor in the higher MICs seen in our AST of *Candida* biofilms.

Antifungal susceptibility of BDPC. Next, to evaluate the possibility that the high resistance seen in *Candida* biofilms is a phenotypic or a genotypic feature, we performed an AST on BDPC. We collected biofilm cells after 24 and48 h and transferred the cells into wells of a new 96-well plate, changing the cells to the planktonic mode. The antifungal susceptibility of these BDPC was almost identical to that of 48-h planktonic *Candida* suspensions, except for AMB, to which cells were more resistant (Table 3). Thus, the MIC of AMB for BDPC of *C. albicans* ATCC 90028 was 1.87 μg/ml, compared with 0.46 μg/ml for the corresponding 48-h planktonic cultures.

Only a few studies thus far have compared the antifungal susceptibilities of biofilm cells and BDPC (4, 5, 20, 28, 30). Although it is difficult to draw firm conclusions due to differences in the methodologies, antifungal agents, and strains employed in these studies, the general consensus is that antifungal

resistance is a phenotypic feature rather than a genotypic alteration of biofilm cells. However, it is noteworthy that some workers have shown that there is a small subpopulation of biofilm cells known as persisters with higher antifungal resistance than the rest of the biofilm population (15, 20, 22). Hence, the marginal increase in the resistance of BDPC to antifungals observed in our study may be due to the presence of these persisters or another trait acquired through the biofilm life-style.

In conclusion, we have carried out a comprehensive study of comparative testing of the antifungal susceptibility of planktonic cells, biofilm cells, and BDPC to five antifungals in clinical use. Our study revealed that NCCLS or EUCAST determinations alone are insufficient to obtain a holistic picture of antifungal susceptibility, especially when the organisms are in the biofilm mode. Therefore, it is imperative to determine the MICs of antifungals at a higher cell density of the planktonic and biofilm modes. Data from such a protocol will perhaps be more useful particularly in clinical settings to select an appropriate antifungal. Furthermore, it could be concluded that although physical factors such as cell density, cellular aging, and growth medium may partially contribute to the higher antifungal resistance seen in *Candida* biofilms, there are other factors related to the biofilm mode which determine its higher antifungal resistance. Current studies with newer molecular biology tools and genomic and proteomic approaches should elucidate the intriguing and as-yet-undiscovered aspects of the antifungal resistance of candidal biofilms.

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